ELSEVIER

Pharmaceutica Acta Helvetiae 74 (1999) 1–9

PHARMACEUTICA ACTA HELVETIAE

www.elsevier.com/locate/pharmactahelv

Stability of L-asparaginase: an enzyme used in leukemia treatment

A.L. Stecher [a], P. Morgantetti de Deus [a], I. Polikarpov [b], J. Abrahão-Neto [a,*]

[a] *Department of Biochemical and Pharmaceutical Technology, Pharmaceutical Sciences School, University of São Paulo, P.O. Box 66083 CEP 05315-970, São Paulo SP, Brazil*

[b] *LNLS-Brazilian National Synchrotron Light Source, P.O. Box 6192 CEP 13083-970, Campinas SP, Brazil*

Received 15 March 1999; received in revised form 17 May 1999; accepted 25 May 1999

Abstract

L-asparaginase from *Escherichia coli* is an important enzyme widely used in leukemia treatment under the trade name Elspar®. Up to now, however, the aspects of its stability and storage has not been studied in detail. The aim of this work is to analyze the factors that could interfere in the enzyme's stability. The enzymatic activity was found to be stable in wide pH range (4.5–11.5), showing a slight increase in activity and stability in alkaline pHs, which indicates a more stable conformation of the molecule. The enzyme proved to have a high activity restoration capacity when submitted to temperatures of 65°C, in pH 8.6 buffer and, surprisingly, in physiologic solution. This suggests a positive effect of sodium ions on such restoration capacity. Stability was high in different diluents used as parenteral solutions and in recipients used in medical practice without significant loss of activity for at least 7 days. These results lead us to conclude that the enzyme has a high stability after the lyophilized form has been reconstituted (at least 7 days), since the necessary precautions are taken in terms of sterile manipulation and if it is stored in a suitable parenteral vehicle under low temperature (about 8°C).

Keywords: L-asparaginase; Leukemia; Stabilization; Storage

1. Introduction

Bacterial L-asparaginases (L-asparagine amidohydrolase, E.C. 3.5.1.1) are enzymes of high therapeutic value due their use in leukemia treatment. *Escherichia coli* L-asparaginase, a high affinity periplasmic enzyme is particularly effective in certain kinds of cancer therapies. A number of bacteria possess L-asparaginase, although not all of these enzymes have anti-tumour properties. The variation in anti-tumour activity has been related to the affinity of the enzyme for its substrate and the clearance rate of the particular types of enzymes. Commercially used enzymes are obtained from *E. coli* and *Erwinia carotovora* (Marlborough et al., 1975).

L-asparaginase was introduced in the therapeutics due to the fact that in a significant number of patients with acute leukemia, particularly lymphocytic, the malignant cells are dependent on a exogenous source of L-asparagine for survival. Normal cells, however, are able to synthesize L-asparagine and thus are less affected by its rapid depletion produced by treatment with the enzyme L-asparaginase. The general medical approach to leukemia therapy is therefore based on a metabolic defect in L-asparagine synthesis of some malignant cells (Broome, 1981; Ravindranath et al., 1992). The enzyme also inhibits protein synthesis by L-asparagine hydrolysis (Marlborough et al., 1975; Ravindranath et al., 1992; Moola et al., 1994). Its action upon DNA and RNA synthesis has not been entirely elucidated yet, but it is believed to be G1-phase specific (Broome, 1981).

The most common therapeutic indications of L-asparaginase are: treatment of Hodgkin disease, treatment

* Corresponding author. Fac. Ciências Farmacêuticas/USP, Av. Prof. Lineu Prestes, 580-Bloco 16, 05508-900, Cidade Universitária, São Paulo, Brazil. Fax: +55-11-815-6386; E-mail: josaneto@usp.br

0031-6865/99/$ - see front matter
PII: S0031-6865(99)00009-6

of acute lymphocytic leukemia (mainly in children), acute myelocytic leukemia, acute myelomonocytic leukemia and chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosarcoma (Cunningham et al., 1979; Levine et al., 1983; Capizzi et al., 1984; Ravindranath et al., 1992; Mitchell et al., 1994; Klumper et al., 1995; Larson et al., 1995). The amino acid sequences of several different asparaginases has been reported, including that of *E. coli* enzyme (Wriston, 1985).

Recent crystallographic studies resulted in an X-ray structures of native (Swain et al., 1993) and T89V mutant L-asparaginase from *E. coli* (Palm et al., 1996), of L-asparaginases from *Wolinella succinogenes* (Lubkowski et al., 1996) and glutaminase–asparaginase from *Pseudomonas* 7 A (Lubkowski et al., 1994). The protein appeared to be a tetramer of four identical subunits, with molecular weight of 35 000/subunit, bound mainly by non-covalent forces (Swain et al., 1993).

Structural modifications to improve the clearance and therapeutic properties have been performed, so that the modified enzyme has a plasmatic half-life longer than 24 h (Harms et al., 1991; Derst et al., 1992; Wehner et al., 1992; Derst et al., 1994). The commercially used enzyme (Elspar®) is isolated from *E. coli* and supplied in a vial of 10 ml with 10 000 UI of the lyophilized enzyme. An important pharmacological characteristic of Erwinase® (Porton-Down, Salisbury, Wiltshire, UK) isolated from *Erw. carotovora* is that this enzyme has no immunological cross-reaction with preparations derived from *E. coli* and, therefore can be used in patients that are hypersensitive to *E. coli* L-asparaginase (Moola et al., 1994).

The purpose of the present work is to investigate L-asparaginase stability and activity changes in different solutions and buffers in respect to the variations of pH and temperature in a wide range. The enzyme is normally supplied in a lyophilized form and, therefore, determination of its proper storage conditions after reconstitution is of vital importance for preservation of its high therapeutic effectiveness and medication.

2. Experimental

2.1. Materials

L-asparaginase was obtained in the form of the concentrate Elspar® with the lyophilized enzyme (Merck, Sharp and Dohme, Pensilvânia, E.U.A.) in vials of 10 ml with

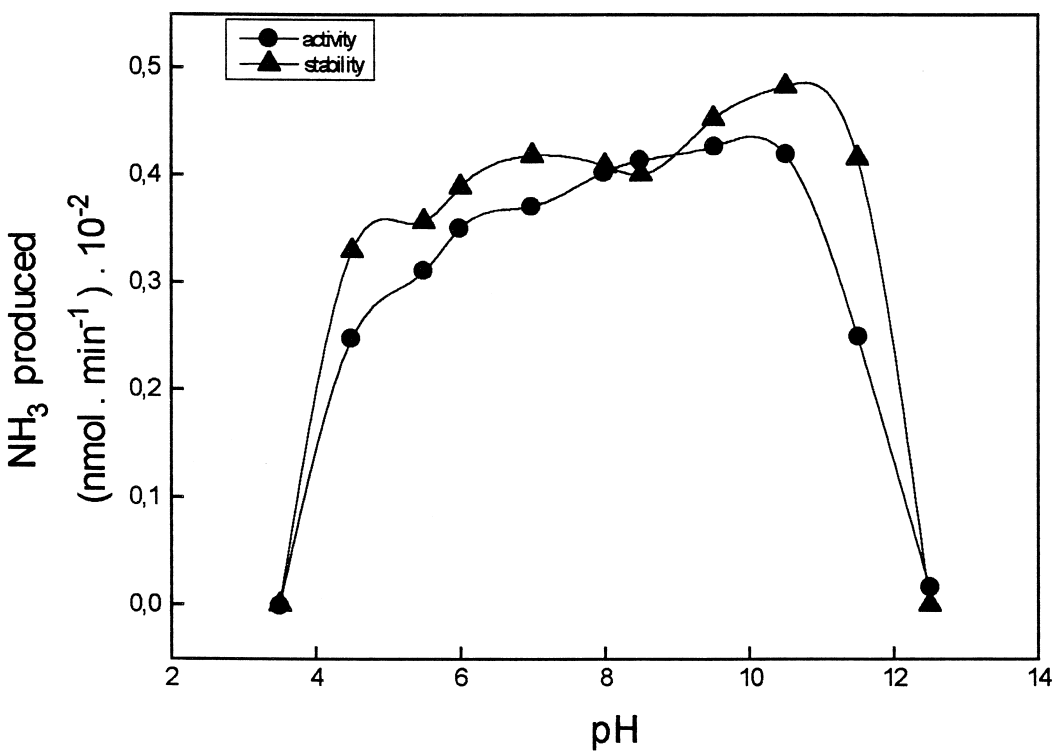

Fig. 1. Enzymatic activity and stability of L-asparaginase. The activity was determined by incubation in different buffers varying the pH from 3.5 to 12.5, (3.5 to 5.5 acetate 0.05 M; 6.0 to 8.0 Hepes 0.05 M and 8.5 to 12.5 Tris/HCl 0.05 M), at 37°C for 10 min. The stability was determined after incubation by 1 h in same buffers at 37°C. At the end of this time the enzymatic activity was determined as described in Section 2 at pH 8.5.

10 000 UI, through Prodome. Serum bags of polyolefin, serum bags of polyethylene (Baxter, B. Brawn, obtained from Essenca Distribuidora de Material Hospitalar). L-asparagine, aspartate, protein mixtures and markers of molecular weight, bovine albumin serum were obtained from Sigma, St. Louis, E.U.A. All the other chemicals were from Merck (Darmstadt, Germany) and were of analytical degree from Quimitra of Brazil, RJ, Brazil.

2.2. Determination of the enzymatic activity

L-asparaginase catalyzes the hydrolysis of L-asparagine producing L-aspartic acid and ammonia. After the adapted time of incubation, in buffer Tris/HCl 50 mM, pH 8.6 containing 10 mM L-asparagine, the reaction was interrupted with 1.5 M of trichloroacetic acid and the samples, after centrifugation, were treated with Nessler reagent. The ammonia concentration produced in the reaction was determined on the basis of a standard curve previously obtained with ammonium sulfate as a standard.

The activity of L-asparaginase can also be measured by using the conductometric method. The method is based on the increase of conductivity, which is due to the production of ammonia and/or aspartate. This conductivity is linear in relation to the time and to the enzymatic concentration and follows Michaelis kinetics (Drainas and Drainas, 1985). The addition of commercial L-asparaginase in the reaction mixture that contains the substrate L-asparagine causes a fast increase in conductivity. This increase is linear to the enzymatic concentration. This method can detect up to 0.001 U of commercial L-asparaginase. Among the advantages of this method are: (a) it is faster because it does not require lengthy preparation and a long reaction time; (b) it is very sensitive; (c) it is reliable due its reproducibility and adapted to the enzyme kinetic study (Drainas and Drainas, 1985). An International Unit (UI) of L-asparaginase is the amount of enzyme that catalyses the production of 1 umol of ammonia liberated in 1 min under the conditions of the assay (Whelan and Wriston, 1969; Law and Wriston, 1971; Wade and Phillips, 1971; Wriston, 1985).

The lyophilized enzyme contained in the medication vial was reconstituted with a suitable parenteral vehicle proposed for study, stored under different conditions (conditions and temperatures proposed) and the enzymatic activity was measured at given intervals of time. Results of

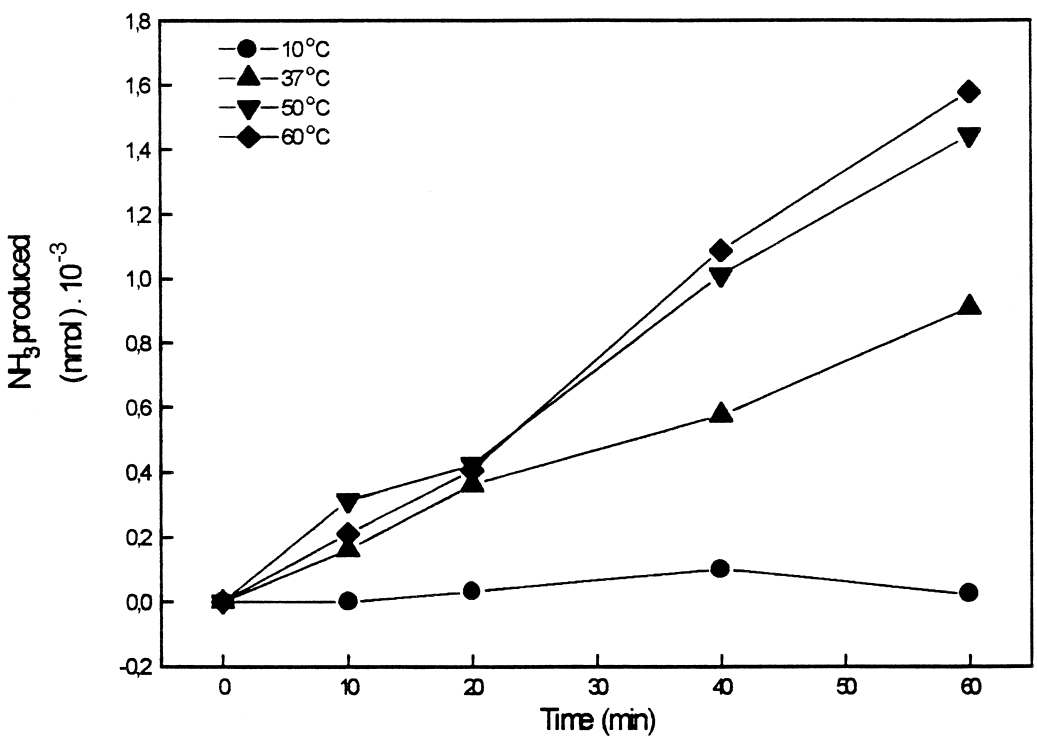

Fig. 2. Activity of L-asparaginase incubated at different temperatures: 10, 37, 50 and 60°C, in buffer Tris/HCl 0.05 M, pH = 8.6 with substrate L-asparagine (100 mM). At the times indicated above, aliquots were removed in order to measure the produced NH_3. Technique of measurements is described in Section 2.

enzymatic activity are the average of three experiments conducted in either a parallel or an independent way.

2.3. Determination of the protein concentration

The concentration of protein was determined by the Bradford Method (Bradford, 1976) using bovine albumin serum as standard. Where necessary, the Lowry Method (Lowry et al., 1951) was used.

3. Results and discussion

In order to verify a purity of the product we conducted an electrophoresis in polyacrylamide gel (SDS-PAGE) (Laemmli, 1970). Analysis of the gel revealed there was no detectable contamination as it represented just one distinct band and the molecular weight of the sub-unit of about 33 kDa, as expected (results not shown).

The analysis of the results of L-ASP activity in different pHs shows that the enzyme has quite a wide range of activity, between 4.5 and 11.5 (Fig. 1). At pH below 4.0 and above 12, the enzyme totally loses its activity. At a pH around 4.5 and 11.5, the activity drops to about 50% of its maximum. After being submitted to these pHs for an hour and then transferred to pH 8.5, enzyme regains activity again. This indicates that at these pHs the conformational changes of the enzyme are still reversible. Also, according to Fig. 1, the profile of enzyme activity shows that it is not constant and there is a slight increase in the activity as pH gets higher. The fact that the activity is maximum in alkaline pH is probably due to the balance between L-aspartic acid and L-aspartate. L-aspartic acid in acid pH has a greater affinity for the active site of the enzyme. Under such conditions, it becomes a competitive inhibitor (Miller et al., 1993). In alkaline pH, the balance is shifted toward the aspartate, which is the form with less affinity to the active site enabling, in this case, a favorable balance for the connection with the substrate L-asparagine (Lubkowski et al., 1994).

Based on the observation of the L-asparaginase, high stability in diverse pH conditions and the its capacity toward activity recovering, the activity tests have been performed at different temperatures in order to study its functional dependence in detail (Fig. 2). In the intervals of time during which the tests were performed (1 h), if one

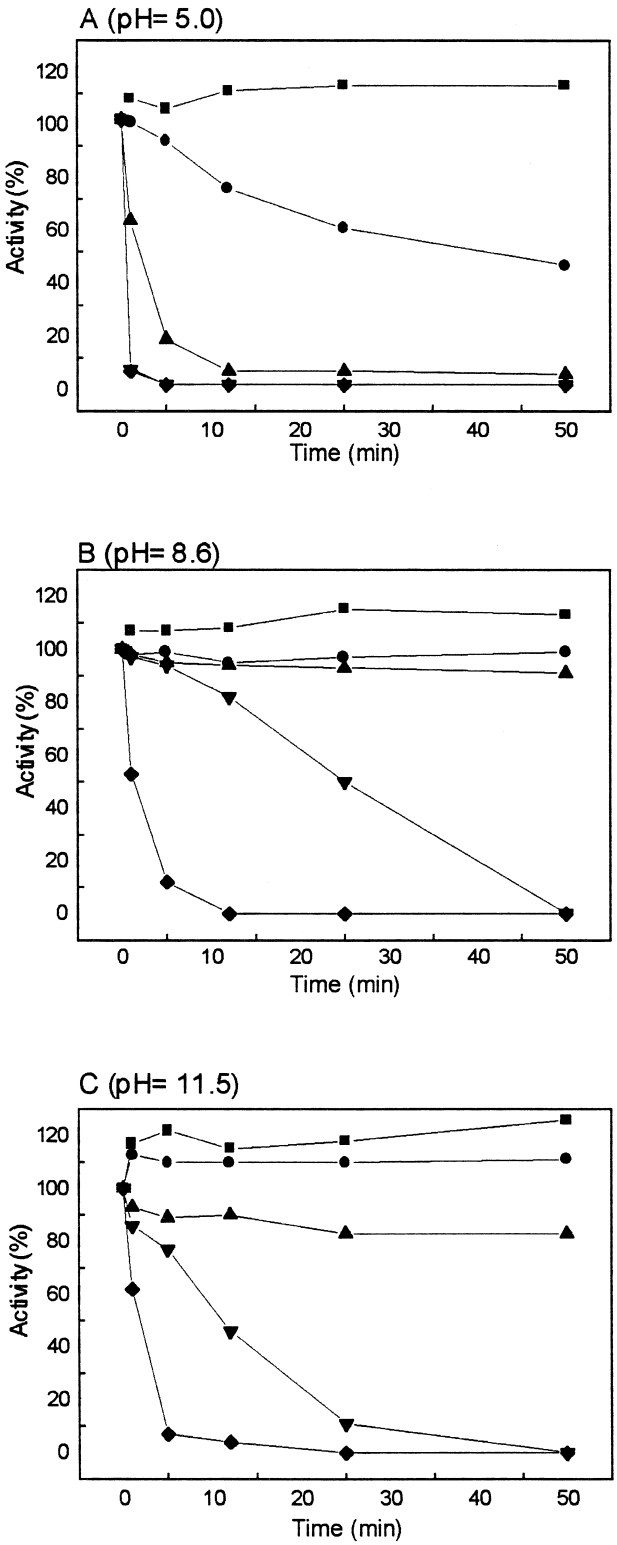

Fig. 3. Thermal stability in different temperatures (■ — 45; ● — 50; ▲ — 55; ▼ — 60; ♦ — 65°C) and pH 5.5 (A), 8.6 (B), and 11.5 (C). After incubation under these conditions, at the indicated times aliquots were withdrawn and assay of the activity was performed as described in Section 2.

will take the activity at 37°C as a reference (100%), the enzyme activity at 10°C was only 10%. At 50°C and 60°C, the enzyme activity was 163% and 186%, respectively, and, almost constant throughout the interval.

When analyzed under different temperatures (45°C, 50°C, 55°C, 60°C and 65°C) and different pHs (5.0, 8.6 and 11.5) (Fig. 3), we observe that in pH 5.0 L-asparaginase is more susceptible to heat if compared to a treatment in pH 8.6. At 50°C, the enzyme submitted to pH 5.0 presents virtually the same profile as the enzyme submitted to 60°C in pH 8.6. Comparing the profiles of the treated enzymes in pH 8.6 and 11.5, we see that they are similar at lower temperatures (up to 50°C). At 60°C, there is a stronger denaturant effect in pH 11.5 than in pH 8.6. In Fig. 3, one notices that the enzyme has a higher resistance in higher pHs (11.5) when compared to acidic pH 5.0 under the same temperatures. These results suggest that in alkaline pHs the enzyme not only adapts more active conformation (Fig. 1), but is also more stable.

It is known that L-asparaginase can completely lose its activity and, also, recover it partially, depending on the exposure conditions. We tried to verify the return of the activity (restoration) after thermal treatment and thus verify a possible similarity between the effect observed in the treatment with urea (Marlborough et al., 1975) and the effects caused by temperature increase on L-asparaginase restoration activity (Fig. 4). We observed that in the studied pHs, L-asparaginase loses its activity totally after the exposure time has elapsed, and after 60 min, at 37°C, the enzyme sample treated in pH 8.6 recovers 50% of the initial activity, while the samples treated with pH 5.0 and 11.5 buffers lose their activity completely, beyond recovery. According to Marlborough et al., 1975, L-asparaginase can be structurally renaturated in alkaline pH but there is no recovery of the activity. These results demonstrate that there was an irreversible structure change in pH 5.0 and 11.5 without restoration that prevents it from hydrolyzing L-asparagine into L-aspartate, while in pH 8.6 there is an active renaturation. This denaturation can be a consequence of the dissociation of the L-asparaginase sub-units. It is important to notice that the activity of this enzyme would be then dependent upon the connections among the subunits, because if each sub-unit has its own active site, they could retain activity when dissociated.

X-ray studies of L-asparaginase from *E. coli* show that the protein is composed of four identical subunits, and this

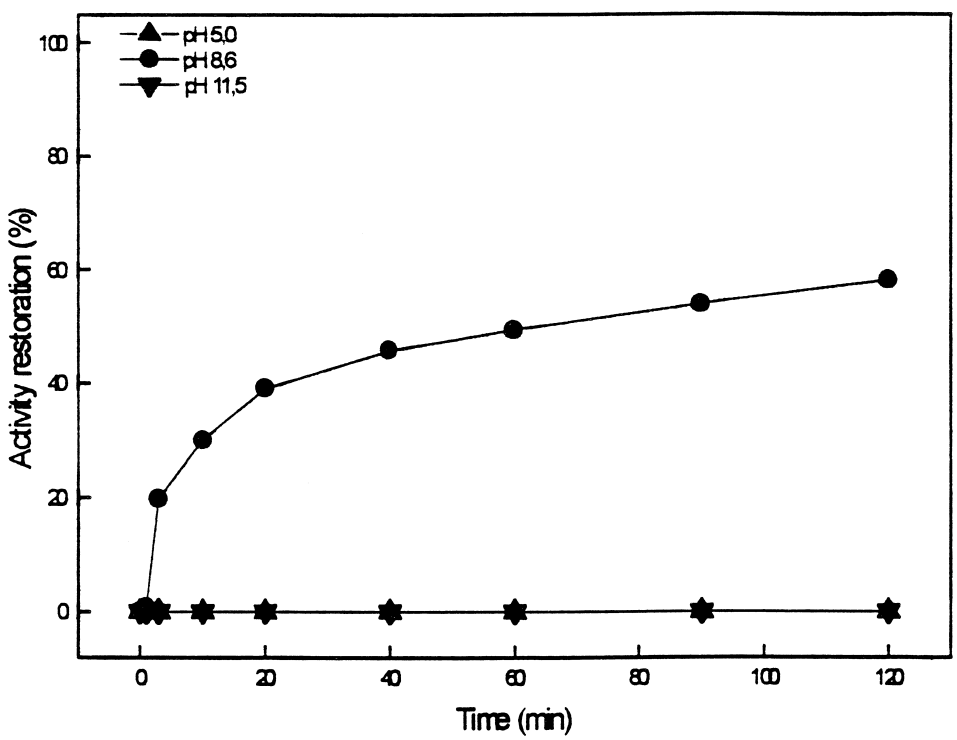

Fig. 4. L-asparaginase activity restoration in different buffers. The enzyme was previously submitted to a thermal treatment in bathing (65°C/20 min). Finished this time, the samples were placed in ice-cold water until 40°C and soon after placed in bathing to 37°C. At the indicated times aliquots were withdrawn and assay of the activity was performed as described in Section 2.

tetramer can be considered as a dimer of intimate dimers (Kraulis, 1991; Swain et al., 1993) (see Fig. 5, structural cartoon). Each of the dimers has two active centers each of them formed by the side chains of the amino acid of both intimately related subunits (Wriston, 1985; Swain et al., 1993; Lubkowski et al., 1994; Lubkowski et al., 1996). Although the dimers contain all the structural elements and functional groups to create a complete active-site environment, the active enzyme is always a tetramer. Calculation of the solvent accessible area of the enzyme (PDB entry code 3eca) done with GRASP shows that upon the formation of the intimate dimer solvent accessible area decreases on 1995.4 Å^2, whereas formation of tetramer from two intimate dimers decreases solvent accessible area on 13 045.5 Å^2. This indicated that although dimer is structurally self-sufficient, a tetramer formation leads to the significant decrease of the free-energy of the enzyme. Apparently, while ionic forces and hydrogen bonds are the main responsible factors for the secondary and tertiary structure, the forces among the subunits in the tetramer are predominantly hydrophobic (Cammack et al., 1972; Shifrin et al., 1973).

These results demonstrate that there is a clear synergism in terms of denaturation and a possibility of renaturation, depending on the temperature and pH. L-asparaginase at pH 8.6 has a good tolerance to heat with return of enzymatic activity (Fig. 4), while in pH 11.5 it has a relatively high thermal stability (Fig. 3). On the other hand, if the

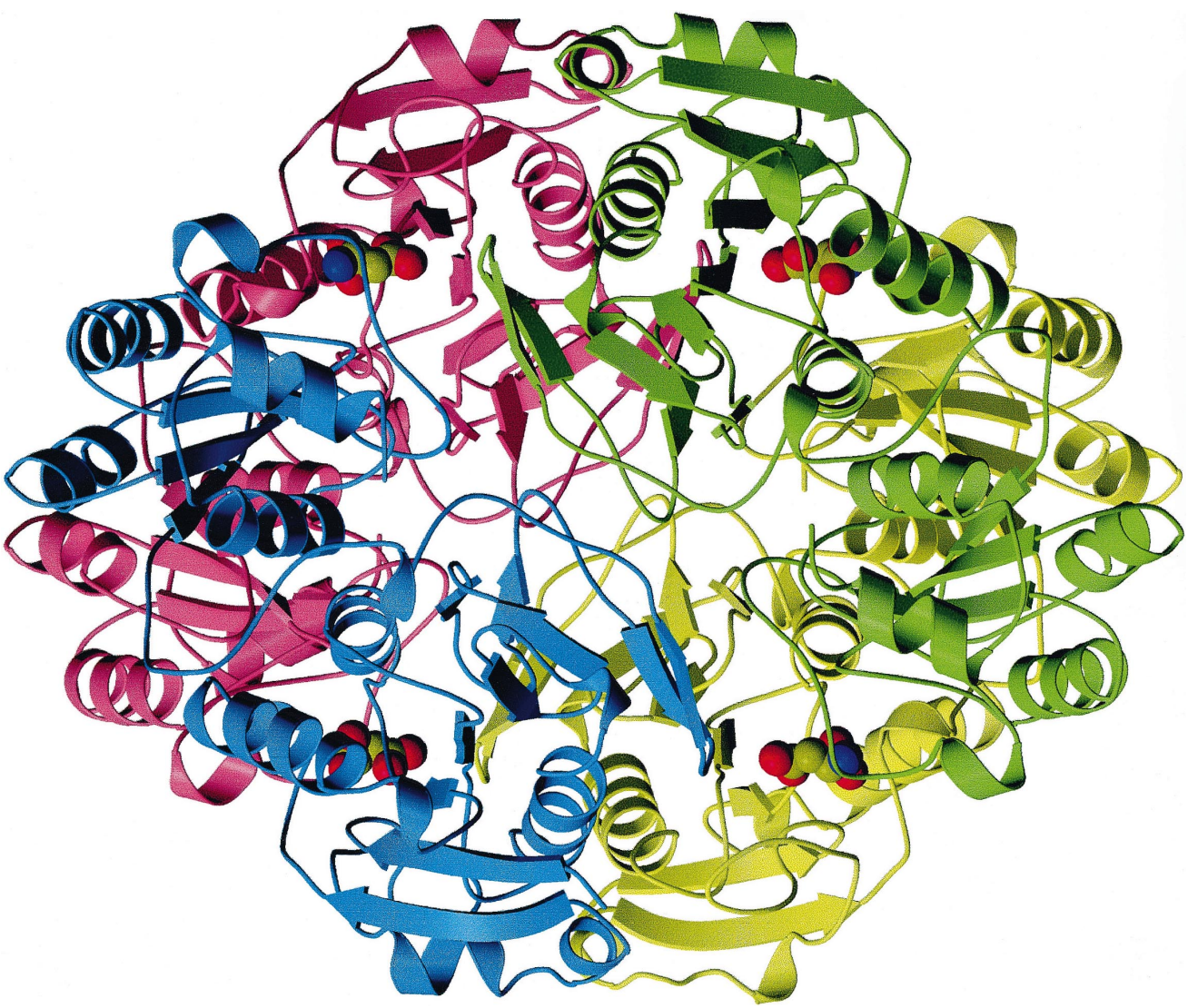

Fig. 5. Structural cartoon of the L-asparaginase tetramer (PDB ID code 3eca) drawn with MOLSCRIPT (33). Monomers are color coded. Products of reaction, aspartates, bound to the active centers of the enzyme is shown as ball-and-stick models.

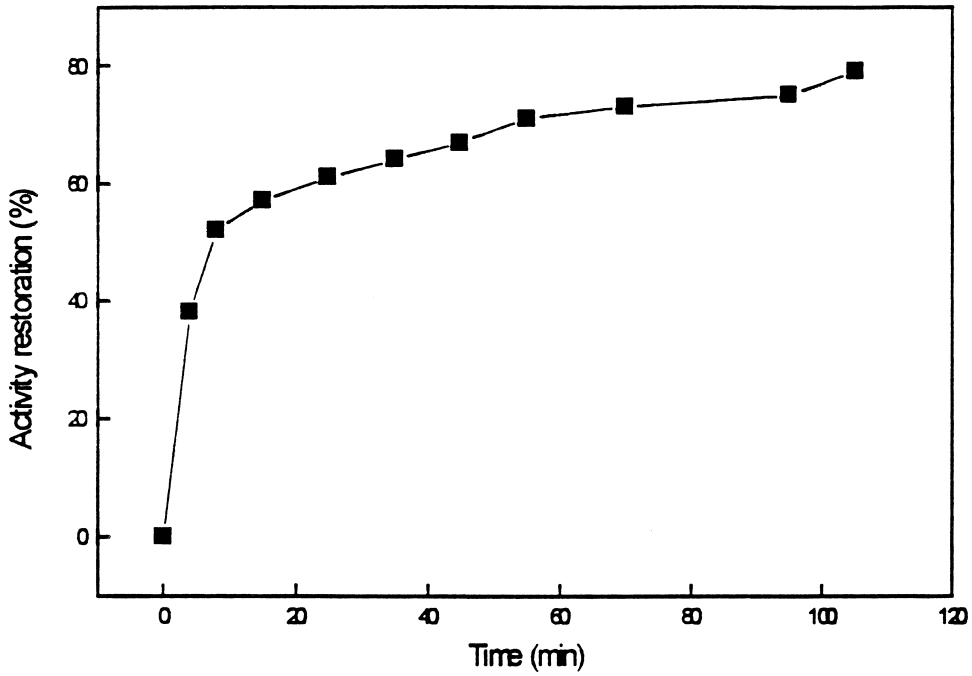

Fig. 6. L-asparaginase activity restoration in physiologic solution. The enzyme was previously submitted to a thermal treatment in bathing (65°C/30 min). Finished this time, the samples were placed in ice-cold water until 40°C and soon after placed in bathing to 37°C. At the indicated times aliquots were withdrawn and assay of the activity was performed as described in Section 2.

enzyme is submitted to pH 11.5 at 65°C, the return of the activity becomes impossible.

To observe a possible restoration of enzyme activity after thermal treatment in physiologic solution, it was

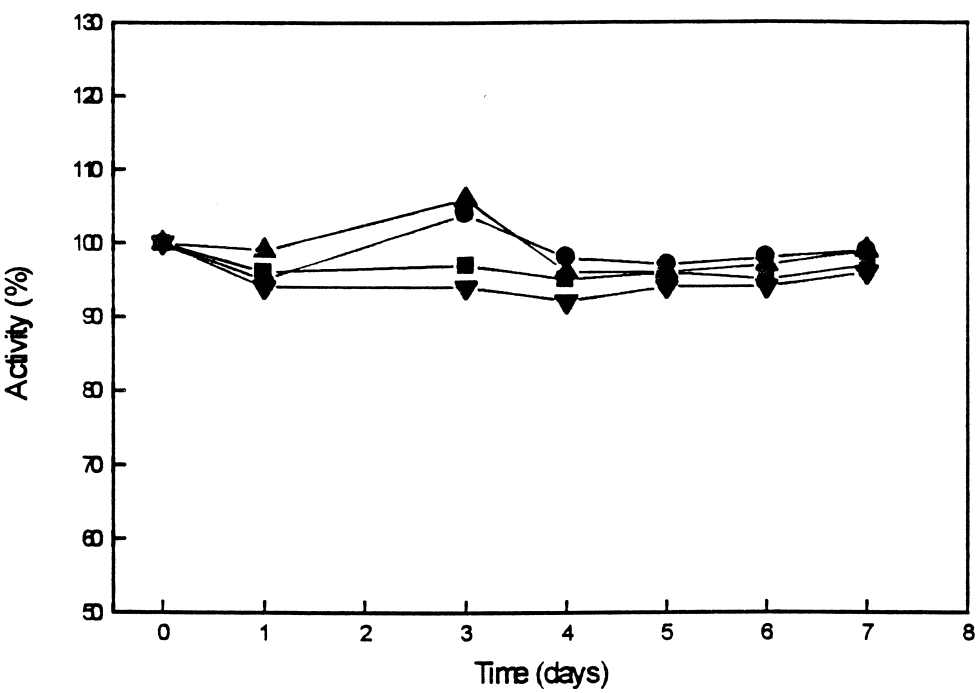

Fig. 7. Stability of L-asparaginase. The enzyme was stored in refrigerator (8°C) in bags of physiologic solution (▲ poly-olefin; ▼ poly-ethylene) and Ringer lactate solution (■ poly-olefin; ● poly-ethylene). At the indicated times aliquots were withdrawn and assay of the activity was performed as described in Section 2.

exposed for 30 min at 65°C in this parenteral solution (Fig. 6). Under these treatment conditions, L-asparaginase totally lost the activity after the exposure period. It is remarkable that within 8 min, the enzyme recovers 50% of the initial activity, that is, there is a strong activity recovery within the first 10 min and after this period of time, the recovery becomes very slow. Comparing the results of activity restoration obtained from the enzyme treated in physiologic solution and in buffers (pH: 5.0; 8.6; 11.5), one verifies that in the first case, after 8 min, the enzyme recovers approximately 50% while in the second condition, recovery is only 30%, that is, the speed of the activity recovery in physiologic solution is significantly higher than in Tris buffer (pH 8.6). In addition, considering that the treatment in physiologic solution was 30 min while in buffer it was only 20 min, a possible positive effect of ions, probably sodium, becomes evident in activity recovery. Experiments show that sodium has a protecting effect on the denaturation of L-asparaginase (Ryoyama, 1972).

As was discussed earlier, the hydrophobic interactions are important for the maintenance of the quaternary structure of L-asparaginase. There seems to be a correlation between the decrease of the enzyme stability and the presence of chaotropic agents, such as KCNS and urea. The our results indicate that sodium, present in the physiologic solution, also favors the enzymatic renaturation.

To verify the stability of L-asparaginase through time (days) in different parenteral solutions and recipients (physiologic solution, ringer-lactate solution in polyolefin bags (soft plastic), polyethylene bags (hard plastic)), the experiments shown in Fig. 7 were performed. The enzyme proved to be stable for a period of 7 days, and the activity drop was very small (about 8%). Analyzing the enzyme behavior in different recipients, we found out that there is no significant interference of the recipient material or the solution studied on stability during this period. It can be noted that L-asparaginase has great stability when stored in a refrigerator (8°C) and in a sterile environment.

The results obtained in this work demonstrate that the enzyme L-asparaginase has a relatively high stability toward the changes in pH (4.5 up to 11.5) and temperature with the possibility of enzyme activity restoration. L-asparaginase keeps its activity for at least 7 days provided that due precautions are taken in terms of sterile manipulation and if stored in suitable parenteral vehicle under low temperature (about 8°C).

Acknowledgements

The authors gratefully acknowledge financial support from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We are grateful to Luís Augusto Angelotti Meira for his help with graphical representation of L-asparaginase crystallographic structure.

References

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

Broome, J.D., 1981. L-asparaginase: discovery and development as a tumor-inhibitory agent. Cancer Treat. Rep. 65, 111–114.

Cammack, K.A., Marlborough, D.I., Miller, D.S., 1972. Physical properties and subunit structure of L-asparaginase isolated from Erwinia carotovora. Biochem. J. 126, 361–379.

Capizzi, R.L., Poole, M., Cooper, M.R., Richards, F., Stuart, J.J., Jackson, D.V., White, D.R., Spurr, C.L., Hopkins, J.O., Muss, H.B., 1984. Treatment of poor risk acute leukemia with sequential high-dose ARA-C and asparaginase. Blood 63, 694–700.

Cunningham, I., Gee, T., Dowling, M., Chaganti, R., Bailey, R., Hopfan, S., Bowden, L., Turnbull, A., Knapper, W., Clarkson, B., 1979. Results of treatment of Ph′ + chronic myelogenous leukemia with an intensive teatment regimen (l-5 protocol). Blood 53, 375–395.

Derst, C., Hensenling, J., Rohm, K.H., 1992. Probing the role of threonine and serine residues of *E. coli* asparaginase II by site-specific mutagenesis. Protein Eng. 5, 785–789.

Derst, C., Wehner, A., Specht, V., Rohm, K.H., 1994. States and functions of tyrosine residues in *Escherichia coli* asparaginase II. Eur. J. Biochem. 224, 553–540.

Drainas, D., Drainas, C., 1985. A conductimetric method for assaying asparaginase activity in *Aspergillus nidulans*. Eur. J. Biochem. 151, 591–593.

Harms, E., Wehner, A., Aung, H.P., Rohm, K.H., 1991. A catalytic role for threonine-12 of *E. coli* asparaginase II as established by site-directed mutagenesis. FEBS Lett. 285, 55–58.

Klumper, E., Pieters, R., Veerman, A.J., Huismans, D.R., Loonen, A.H., Hahlen, K., Kaspers, G.J., Van Wering, E.R., Hartmann, R., Henze, G., 1995. In vitro cellular drug resistence in children with relapsed/refractory acute lymphoblastic leukemia. Blood 86, 3861–3868.

Kraulis, P.J., 1991. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. 24, 946–950.

Laemmli, U.K., 1970. Cleavage of strutural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Larson, R.A., Dodge, R.K., Burns, C.P., Lee, E.J., Stone, R.M., Schulman, P., Duggan, D., Davey, F.R., Sobol, R.E., Frankel, S.R., 1995. A five-drug remission induction regimen with intensive consolidation for adults with limphoblastic leukemia: cancer and leukemia group B study 8811. Blood 85, 2025–2037.

Law, A.S., Wriston, J.C., 1971. Purification and properties of *Bacillus coagulans* L-asparaginase. Arch. Biochem. Biophys. 147, 744–752.

Levine, A.M., Forman, S.J., Meyer, P.R., Koehler, S.C., Liebman, H., Paganini-Hill, A., Pockros, A., Lukes, R.J., Feinstein, D.I., 1983. Successful therapy of convoluted T-lymphoblastic lymphoma in the adult. Blood 61, 92–98.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lubkowski, J., Wlodawer, A., Ammon, H.L., Copeland, T.D., Swain, A.L., 1994. Structural characterization of *Pseudomonas* 7A glutaminase-asparaginase. Biochemistry 33, 10257–10265.

Lubkowski, J., Palm, G.J., Giliand, G.L., Derst, C.K., Rohm, H., Wlodawer, A., 1996. Crystal structure and amino acid sequence of *Wolinella succinogenes* L-asparaginase. Eur. J. Biochem. 241, 201–207.

Marlborough, D.I., Miller, D.S., Cammack, K.A., 1975. Comparative study on conformational stability and subunit interactions of two bacterial asparaginases. Biochim. Biophys. Acta 386, 576–589.

Miller, M., Mohana Rao, J.K., Wlodawer, A., Gribskov, M.R., 1993. Crystal structure of *Erwinia chrysanthemi* L-asparaginase with bound L-aspartate. FEBS Lett. 328 (3), 275–279.

Mitchell, L., Hoogendoorn, H., Giles, A.R., Vegh, P., Andrew, M., 1994. Increased endogenous thrombin generation in children with acute lymphoblastic leukemia: risk of thrombotic complications in L'Asparaginase-induced antithrombin III deficiency. Blood 83, 386–391.

Moola, Z.B., Scawen, M.D., Atkinson, T., Nicholls, D.J., 1994. *Erwinia chrysanthemi* L-asparaginase: epitope mapping and production of antigenically modified enzymes. Biochem. J. 302, 921–927.

Palm, G.J., Lubkowski, J., Derst, C., Schleper, S., Rohm, K.-H., Wlodawer, A., 1996. A covalently bound catalytic intermediate in *Escherichia coli* asparaginase: crystal structure of a Thr-89-Val mutant. FEBS Lett. 390, 211–216.

Ravindranath, Y., Abella, E., Krischer, J.P., Wiley, J., Inoue, S., Harris, M., Chauvenet, A., Alvarado, C.S., Dubowy, R., Ritchey, A.K., Land, V., Stueber, C.P., Weinstein, H., 1992. Acute myeloid leukemia (AML) in Down's syndrome is highly responsive to chemotherapy: experience on Pediatric Oncology Group AML Study 8498. Blood 80, 2210–2214.

Ryoyama, C., 1972. Nature of protective action of sodium and potassium ions against heat inactivation of *Escherichia coli* L-asparaginase. Biochim. Biophys. Acta 26, 539–541.

Shifrin, S., Solis, B.G., Chaiken, I.M., 1973. L-asparaginase from *Erwinia carotovora*. Physicochemical properties of the native and succinylated enzyme. J. Biol. Chem. 248, 3464–3469.

Swain, A.L., Jaskolski, M., Housset, D., Mohana Rao, J.K., Wlodawer, A., 1993. Crystal struture of *E. coli* asparaginase, an enzyme used in cancer therapy. Proc. Natl. Acad. Sci. U.S.A. 90, 1474–1478.

Wade, H.E., Phillips, B.P., 1971. Automated determination of bacterial asparaginase and glutaminase. Anal. Biochem. 44, 189–199.

Wehner, A., Harms, E., Jennings, M.P., Beacham, I.R., Derst, C., Bast, P., Rohm, K.H., 1992. Site-specific mutagenesis of *Escherichia coli* asparaginase: II. None of the three histidine residues is required for catalysis. Eur. J. Biochem. 208, 475–480.

Whelan, H.A., Wriston, J.C., 1969. Purification and properties of asparaginase from *Escherichia coli* B. Biochemistry 8, 2386–2393.

Wriston, J.C., 1985. Asparaginase. Methods Enzymol. 113, 608–618.